

## CUTICULAR HYDROCARBONS OF *Drosophila birchii* AND *D. serrata*: IDENTIFICATION AND ROLE IN MATE CHOICE IN *D. serrata*

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**Abstract**—The cuticular hydrocarbon compositions of two sympatric species of Australian *Drosophila* in the *montium* subgroup of the *melanogaster* group that use cuticular hydrocarbons in mate recognition have been characterized. *Drosophila birchii* has 34 components in greater than trace amounts, with a carbon number range of C<sub>20</sub> to C<sub>33</sub>. *Drosophila serrata* has 21 components above trace level and a carbon number range of C<sub>24</sub> to C<sub>31</sub>. These two species share eight hydrocarbon components, with all but two of them being monoenes. For both species, the (Z)-9-monoenes are the predominant positional isomer. The hydrocarbons of *D. birchii* are *n*-alkanes, *n*-alkenes (Z)-5-, (Z)-7-, (Z)-9-, and (Z)-11-, low to trace levels of homologous (Z,Z)-7,11- and (Z,Z)-9,13-dienes; and trace amounts of (Z,Z)-5,9-C<sub>25,2</sub>, a major component of *D. serrata*. Only one methyl branched hydrocarbon was detected (2-methyl C<sub>28</sub>), and it occurred at very low levels. The hydrocarbons of *D. serrata* are dominated by a homologous series of (Z,Z)-5,9-dienes, and notably, are characterized by the apparent absence of *n*-alkanes. Homologous series of (Z)-5-, (Z)-7-, and (Z)-9-alkenes are also present in *D. serrata* as well as 2-methyl alkanes. *Drosophila serrata* females display strong directional mate choice based on male cuticular hydrocarbons and prefer *D. serrata* males with higher relative abundances

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of the 2-methyl alkanes, but lower relative abundances of (Z,Z)-5,9-C<sub>24:2</sub> and (Z)-9-C<sub>25:1</sub>.

**Key Words**—Hydrocarbons, pheromones, sexual selection, sibling species, mass spectra, dienes, monoenes, 2-methylalkanes.

## INTRODUCTION

In *Drosophila*, and in particular in members of the *melanogaster* species group, cuticular hydrocarbons have been shown to contribute to mate choice within species (Jallon, 1984) and sexual isolation between species (Cobb and Jallon, 1990; Coyne et al., 1994; Buckley et al., 1997; Coyne and Charlesworth, 1997). *Drosophila birchii* and *D. serrata* are two closely related species of the *montium* subgroup of the *melanogaster* group. Females of the two species are morphologically identical, and males are distinguishable only by the numbers of hairs on their genital arch (Ayala, 1965). Despite these morphological similarities, the species are sexually isolated (Ayala, 1965; Blows and Allan, 1998). Four lines of evidence suggest that cuticular hydrocarbons play an important role in mate recognition in the *D. serrata* complex. First, perfuming experiments (where cuticular chemicals are transferred between flies) indicated that olfaction may be an important component involved in sexual isolation (Blows and Allan, 1998). Second, the high genetic correlation between mate recognition and cuticular hydrocarbons in hybrid populations (Blows and Allan, 1998) suggested that these chemicals may be the major mechanism by which mate recognition evolves. Third, reproductive character displacement of these hydrocarbons has evolved in populations of *D. serrata* that are sympatric with *D. birchii*, demonstrating that cuticular hydrocarbons are involved in species recognition under natural conditions (Higgie et al., 2000). Finally, hybrid male cuticular hydrocarbons rapidly responded to the manipulation of sexual selection over 20 generations (Blows, 2002).

Cuticular hydrocarbons from many species of the *melanogaster* subgroup have been analyzed, and in some species cuticular hydrocarbon sexual dimorphism exists (Jallon and David, 1987). Several species of the *melanogaster* group produce alkadienes in significant amounts (Jallon, 1984; Cobb and Jallon, 1990). The alkadiene (Z,Z)-7,11-heptacosadiene in *D. melanogaster* females stimulates a dose-dependent courtship and mating response from males (Ferveur and Sureau, 1996); however, (Z,Z)-5,9-heptacosadiene, which is also found on females, does not induce courtship from male *D. melanogaster* in doses normally found on sexually mature females. Although previous work on the *D. serrata* complex has provided evidence that *D. birchii* and *D. serrata* use cuticular hydrocarbons as species recognition cues, and possibly as sex pheromones within species, the individual hydrocarbons that may be involved have not been characterized. These two species are the first members of the *montium* subgroup of the *melanogaster* group to have their cuticular hydrocarbons reported

in detail. In addition, we provide experimental evidence that *D. serrata* females choose *D. serrata* males based on at least four of these cuticular hydrocarbons.

#### METHODS AND MATERIALS

*Insects.* One population of *D. birchii* from Eungella and one population of *D. serrata* from Byfield in north Queensland, Australia, were used to characterize individual hydrocarbons. The Forster population of *D. serrata* (described in Higgin et al., 2000) was used in the mate choice experiment. All cultures were maintained in the laboratory for 1–3 years with census sizes >300 individuals.

*Chemical Analysis.* Hydrocarbons of 4-day-old virgin females and males of both species were separately extracted in hexane, concentrated to dryness under nitrogen, and shipped in Teflon-capped microvials to Manhattan, Kansas, USA, where they were held at  $-80^{\circ}\text{C}$  until analyzed. Individual samples were made up with hexane to a volume of 10  $\mu\text{l}$  and analyzed by capillary gas chromatography (GC)–mass spectrometry (MS) in both the electron impact (EI) and methane chemical ionization (CI) mode. Mass spectral analyses were conducted using a Hewlett-Packard 5790A gas chromatograph (GC) (Hewlett-Packard, Inc., San Fernando, California, USA) containing a DB-5 bonded-phase capillary column (15 m long, 0.25 mm inside diam.) (J and W Scientific, Folsom, California, USA) connected to a Hewlett-Packard 5970 mass selective detector (MSD) and a Hewlett-Packard 9133 data system. Ultrapure helium was the carrier gas, with a column head pressure of 0.75  $\text{kg}/\text{cm}^2$ . Mass spectra were obtained at 70 eV. Analyses were done using temperature programming, with an initial temperature of  $150^{\circ}\text{C}$ , a final temperature of  $320^{\circ}\text{C}$ , a program rate of  $5^{\circ}\text{C}/\text{min}$ , and a 20-min final hold period. The splitless injector was set at  $275^{\circ}\text{C}$  and the GC-MSD interface was at  $280^{\circ}\text{C}$ . Retention times of each hydrocarbon component and equivalent chain length values (ECL) were obtained by comparison with known *n*-alkane standards (Howard et al., 1978). Individual components in the total ion-scanning mode were identified from their characteristic EI- and CI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978; Howard et al., 1980; Harrison, 1983). Double-bond locations in alkenes and alkadienes were obtained by preparing dithiomethyl ethers and examining their EI mass spectra (Francis and Veland, 1981; Vicenti et al., 1987). We were unsuccessful in analyzing the dienes of *D. birchii* as thiomethyl ethers, and their structures were determined by EI-MS fragmentation analysis and retention time data of the parent diene only. Stereochemistry of the parent alkenes and alkadienes were established from Fourier transform infrared (FT-IR) analyses. The FT-IR vapor-phase spectra on the underivatized compounds were obtained on a Hewlett-Packard 5890 GC with a 5965B FT-IR detector and a 7958A data system. A DBX-5 bonded-phase capillary column (50 m long, 0.32 mm inside diam.) using chromatographic conditions identical to those described above was used.

For hydrocarbon quantification, newly eclosed flies were separated by sex and aged to 5 days of age in groups of 10–20 single sex flies. Five-day-old flies were extracted in groups of 10 flies and the extract chromatographed on an HP-1 bonded-phase capillary column (12 m long, 0.25 mm inside diam.) programmed from 150°C to 300°C at 5°C/min. Quantification of the hydrocarbons was done by comparing total ion current values to 100 ng/fly of octacosane as an internal standard.

*Mate Choice Experiment.* Individual virgin 6-day-old *D. serrata* females were placed in vials with 2 virgin *D. serrata* males, in a total of 123 such mate choice tests. Females were watched, and when a male successfully mounted, the 2 males were removed from the vial, and samples were individually prepared for analysis by gas chromatography, resulting in a total of 246 individual hydrocarbon profiles. Individual flies were soaked in 50  $\mu$ l hexane for 4 min, agitated on a vortex mixer for 1 min, and 0.2  $\mu$ l was then injected into the gas chromatograph. Gas chromatography was carried out on a Hewlett Packard HP6890 gas chromatograph with a 50-m 0.32-mm-ID BPX5 capillary column. The temperature program was 50 to 220°C at 15°C/min, then 220 to 320°C at 30°C/min followed by a final 15 min hold at 320°C. Ultrapure helium was the carrier gas and detection was via flame ionization.

A multivariate approach to the analysis of cuticular hydrocarbons (Blows and Allan, 1998; Higgie et al., 2000) was employed to determine the overall discrimination between rejected and chosen males, based on nine hydrocarbons. Preliminary examination of the data using up to 15 hydrocarbons had shown that the additional hydrocarbons did not increase the explanatory power of the model. Relative concentrations for the nine hydrocarbons were calculated by dividing each peak's area by the total peak area summed over the nine hydrocarbons. This created a compositional data set where the nine proportions summed to one. To break this unit-sum constraint, the proportions were transformed to logcontrasts (Aitchison, 1986). Logcontrasts were generated by dividing eight of the proportions by an arbitrarily chosen ninth proportion [in this case, that of (Z,Z)-5,9-C<sub>24</sub>] and then taking the log of each of the new variables.

## RESULTS

*Species Differences in Cuticular Hydrocarbons.* Preliminary mass spectral analysis of hexane extracts of *D. birchii* and *D. serrata* indicated no discernible compounds other than cuticular hydrocarbons, so all samples were analyzed without silica gel chromatography. The hydrocarbon profiles of both fly species were relatively simple compared to most insect species, with 34 compounds in greater than trace amounts (ca. 0.2–0.6 ng/fly) for *D. birchii* (Figure 1A) and 21 for *D. serrata* (Figure 1B). For both species, males and females had identical hydrocarbon components, although relative concentration differences were apparent for many compounds. The *D. serrata* extracts contained no discernible *n*-alkanes, and the *D. birchii* had only one 2-methyl alkane. The data used to identify the hydrocarbons of both species are presented in Tables 1 and 2 and calculated

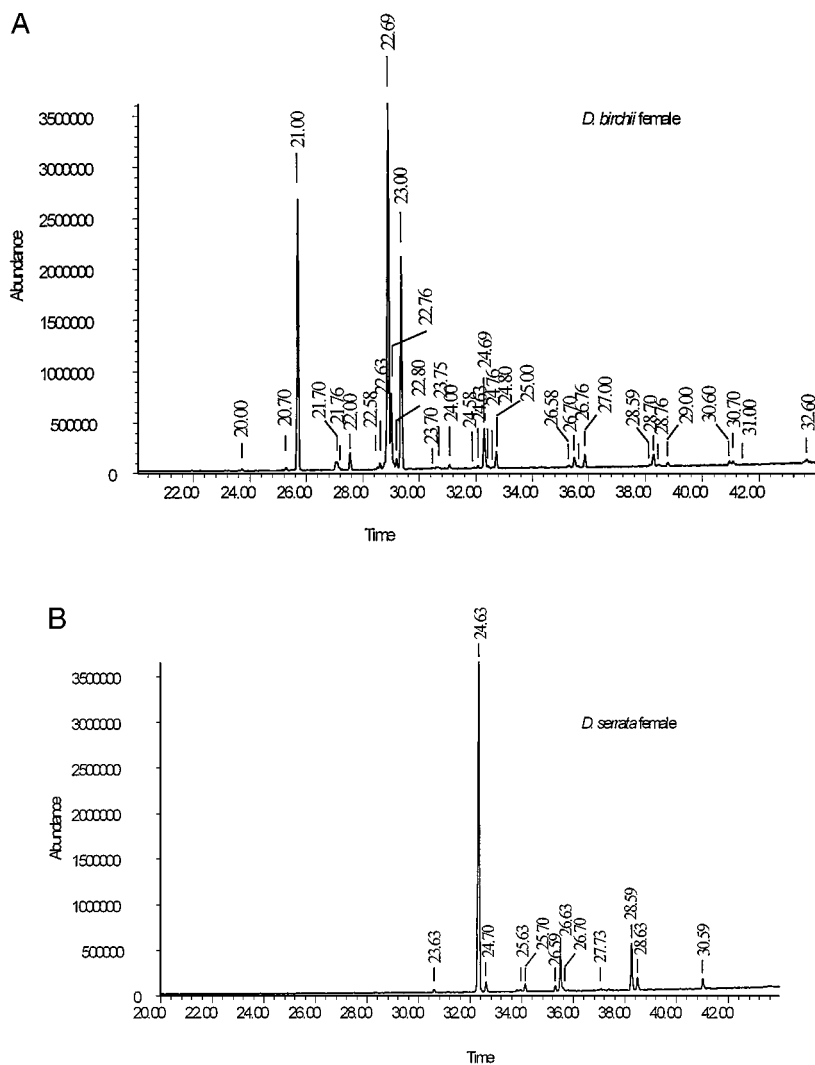


FIG. 1. (A) Total ion trace of the cuticular hydrocarbons of female *D. birchii* (A) and *D. serrata* (B). Each peak is labeled with its equivalent chain length.

equivalent chain length values for each GC peak are also indicated on Figures 1A,B. Quantitative composition data are presented in Tables 3 and 4. FT-IR analysis indicated that all alkenes and alkadienes were of *Z*-configuration [absence of a strong band at  $970\text{ cm}^{-1}$  and presence of a weak band at  $3012\text{ cm}^{-1}$  and a weak to moderate band at  $1650\text{ cm}^{-1}$  (Nakanishi, 1962; Krokos et al., 2001)], as is true for the vast majority of insect unsaturated hydrocarbons (Howard, 1993). The monoenes were

TABLE 1. CUTICULAR HYDROCARBONS OF *Drosophila birchii*

Hydrocarbon	ECL <sup>a</sup>	CN <sup>b</sup>	Diagnostic ions (m/z) <sup>c</sup>
C <sub>20</sub>	20.00	20	282; (281)
(Z)-9-C <sub>21:1</sub>	20.70	21	294; (293, 295); [388, 173, 201]
C <sub>21</sub>	21.00	21	296; (295)
(Z)-9-C <sub>22:1</sub>	21.70	22	308; (307, 309); [402, 173, 215]
(Z)-7-C <sub>22:1</sub>	21.76	22	308; (307, 309); [402, 145, 243]
C <sub>22</sub>	22.00	22	310; (309)
(Z,Z)-7,11-C <sub>23:2</sub>	22.58	23	320, 152, 166, 222, 236; (319)
(Z,Z)-5,9-C <sub>23:2</sub>	22.63	23	320, 124, 138, 250, 264; (319)
(Z)-11-C <sub>23:1</sub>	22.65	23	322; (321, 323); [416, 201, 215]
(Z)-9-C <sub>23:1</sub>	22.69	23	322; (321, 323); [406, 173, 243]
(Z)-7-C <sub>23:1</sub>	22.76	23	322; (321, 323); [416, 145, 271]
(Z)-5-C <sub>23:1</sub>	22.80	23	322; (321, 323); [416, 117, 299]
C <sub>23</sub>	23.00	23	324; (323)
(Z)-9-C <sub>24:1</sub>	23.70	24	336; (337, 339); [430, 173, 257]
(Z)-7-C <sub>24:1</sub>	23.75	24	336; (337, 339); [430, 145, 285]
C <sub>24</sub>	24.00	24	338; (337)
(Z,Z)-7,11-C <sub>25:2</sub>	24.58	25	348, 152, 166, 250, 264; (347)
(Z,Z)-5,9-C <sub>25:2</sub>	24.63	25	348, 124, 138, 278, 292; (347)
(Z)-9-C <sub>25:1</sub>	24.69	25	350; (349, 351); [444, 173, 271]
(Z)-7-C <sub>25:1</sub>	24.76	25	350; (349, 351); [444, 145, 299]
(Z)-5-C <sub>25:1</sub>	24.80	25	350; (349, 351); [444, 117, 327]
C <sub>25</sub>	25.00	25	352; (351)
(Z,Z)-7,11-C <sub>27:2</sub>	26.58	27	376, 152, 166, 278, 292; (375)
(Z)-9-C <sub>27:1</sub>	26.70	27	378; (377, 379); [472, 173, 299]
(Z)-7-C <sub>27:1</sub>	26.76	27	378; (377, 379); [472, 145, 327]
C <sub>27</sub>	27.00	27	380; (379)
(Z,Z)-7,11-C <sub>29:2</sub>	28.59	29	404, 152, 166, 306, 320; (403)
2-MeC <sub>28</sub>	28.59	29	408, 393, 365
(Z)-9-C <sub>29:1</sub>	28.70	29	406; (405, 407); [500, 173, 327]
C <sub>29</sub>	29.00	29	408; (407)
(Z,Z)-7,11-C <sub>31:2</sub>	30.60	31	432, 152, 166, 334, 348; (431)
(Z)-9-C <sub>31:1</sub>	30.70	31	434; (433, 435); [528, 173, 355]
C <sub>31</sub>	31.00	31	436; (435)
(Z,Z)-7,11-C <sub>33:2</sub>	32.60	33	460, 152, 166, 362, 376; (459)

<sup>a</sup> Equivalent chain length. (DB-5 column).<sup>b</sup> Carbon number.<sup>c</sup> Ions in parentheses are diagnostic ions from chemical ionization mass spectral analysis; ions in brackets are of diagnostic ions from thiomethyl ethers.

all readily characterized as to location of double bond position by EI-MS analysis of their dithiomethyl ethers (Francis and Veland, 1981). In common with many other *Drosophila* species (Jallon and David, 1987), for any given carbon number, the alkenes of *D. birchii* and *D. serrata* exist as a mixture of positional isomers, with (Z)-9 being the major isomer for both species.

TABLE 2. CUTICULAR HYDROCARBONS OF *Drosophila serrata*

Hydrocarbon	ECL <sup>a</sup>	CN <sup>b</sup>	Diagnostic ions ( <i>m/z</i> ) <sup>c</sup>
(Z,Z)-5,9-C <sub>24:2</sub>	23.63	24	324, 124, 138, 264, 278; (323); [460, 117, 257, 203, 343]
(Z,Z)-5,9-C <sub>25:2</sub>	24.63	25	348, 124, 138, 278, 292; (347); [474, 117, 271, 203, 357]
(Z)-9-C <sub>25:1</sub>	24.70	25	350; (349, 351); [444, 173, 271]
(Z)-7-C <sub>25:1</sub>	24.76	25	350; (349, 351); [444, 145, 299]
(Z)-5-C <sub>25:1</sub>	24.80	25	350; (349, 351); [444, 117, 327]
(Z,Z)-5,9-C <sub>26:2</sub>	25.63	26	362, 124, 138, 292, 306; (361); [488, 117, 285, 203, 371]
(Z)-9-C <sub>26:1</sub>	25.70	26	364; (363, 365); [458, 173, 285]
(Z)-8-C <sub>26:1</sub>	25.73	26	364; (363, 365); [458, 159, 299]
(Z)-7-C <sub>26:1</sub>	25.76	26	364; (363, 365); [458, 145, 313]
2-MeC <sub>26</sub>	26.59	27	380, 365, 337
(Z,Z)-5,9-C <sub>27:2</sub>	26.63	27	376, 124, 138, 306, 320; (375); [502, 117, 299, 203, 385]
(Z)-9-C <sub>27:1</sub>	26.70	27	378; (377, 379); [472, 173, 299]
(Z)-7-C <sub>27:1</sub>	26.76	27	378; (377, 379); [472, 145, 327]
(Z)-5-C <sub>27:1</sub>	26.80	27	378; (377, 379); [472, 117, 355]
(Z)-8-C <sub>28:1</sub>	27.73	28	392; (391, 393); [486, 159, 327]
(Z)-6-C <sub>28:1</sub>	27.78	28	392; (391, 393); [486, 131, 355]
2-MeC <sub>28</sub>	28.59	29	408, 393, 365
(Z,Z)-5,9-C <sub>29:2</sub>	28.63	29	404, 124, 138, 334, 348; (403); [530, 117, 327, 203, 413]
(Z)-9-C <sub>29:1</sub>	28.70	29	406; (405, 407); [500, 173, 327]
(Z)-7-C <sub>29:1</sub>	28.76	29	406; (405, 407); [500, 145, 355]
2-MeC <sub>30</sub>	30.59	31	436, 421, 393

<sup>a</sup> Equivalent chain length. (DB-5 column).<sup>b</sup> Carbon number.<sup>c</sup> Ions in parentheses are diagnostic ions from chemical ionization mass spectral analysis; ions in brackets are of diagnostic ions from thiomethyl ethers.

Characterization of the location of double bonds in the alkadienes of *D. serrata* was relatively straightforward. When alkadienes have their double bonds separated by four or fewer methylene units, reaction with CH<sub>3</sub>SSCH<sub>3</sub> leads to heterocyclic products with one sulfur atom in the ring, and side chains with thiomethyl ether substituents. The mass spectra of these diastereomeric products have abundant molecular ions and strong fragment ions that are readily interpreted (Vicenti et al., 1987; Howard, 1993) (Figure 2A). All dienes in *D. serrata* had their double bonds at positions 5 and 9 and were of the *Z*-configuration (Table 2).

Although the dienes in *D. birchii* were present in high enough abundance to obtain useful EI and CI mass spectra (Table 1), we were not able to obtain thiomethylation data. Unlike monoenes, where it is well known that location of the double bond position can not be easily assigned from EI mass spectral fragmentation patterns, alkadienes that have their double bonds separated by only a few methylene units do give mass spectral patterns that are associated with double bond location (Descoins et al., 1986; Swedenborg and Jones, 1992). Figure 2B presents the EI-MS profile of (Z,Z)-5,9-C<sub>25:2</sub> obtained from *D. serrata*, with

TABLE 3. HYDROCARBON ABUNDANCE OF 5-DAY OLD  
*D. birchii*

Hydrocarbon	Amount (ng/fly, mean $\pm$ SEM) <sup>a</sup>	
	Female	male
C <sub>20</sub>	tr <sup>b</sup>	tr
(Z)-9-C <sub>21:1</sub>	tr	tr
C <sub>21</sub>	125 $\pm$ 36	127 $\pm$ 40
(Z)-9-; (Z)-7-C <sub>22:1</sub>	5 $\pm$ 1	5 $\pm$ 3
C <sub>22</sub>	4 $\pm$ 1	6 $\pm$ 2
(Z,Z)-5,9-; (Z,Z)-7,11-C <sub>23:2</sub>	3 $\pm$ 2	4 $\pm$ 2
(Z)-11-; (Z)-9-C <sub>23:1</sub>	311 $\pm$ 92	164 $\pm$ 44
(Z)-7-C <sub>23:1</sub>	44 $\pm$ 17	30 $\pm$ 9
(Z)-5-C <sub>23:1</sub>	5 $\pm$ 3	1 $\pm$ 1
C <sub>23</sub>	110 $\pm$ 21	90 $\pm$ 27
(Z)-9-; (Z)-7-C <sub>24:1</sub>	tr	tr
C <sub>24</sub>	tr	tr
(Z,Z)-5,9-; (Z,Z)-7,11-C <sub>25:2</sub>	2 $\pm$ 1	4 $\pm$ 2
(Z)-9-; (Z)-7-; (Z)-5-C <sub>25:1</sub>	12 $\pm$ 2	7 $\pm$ 4
C <sub>25</sub>	17 $\pm$ 6	25 $\pm$ 4
(Z,Z)-7,11-C <sub>27:2</sub>	5 $\pm$ 2	1 $\pm$ 1
(Z)-9-C <sub>27:1</sub>	13 $\pm$ 4	3 $\pm$ 1
(Z)-7-C <sub>27:1</sub>	3 $\pm$ 1	5 $\pm$ 1
C <sub>27</sub>	23 $\pm$ 6	18 $\pm$ 3
(Z,Z)-7,11-C <sub>29:2</sub>	14 $\pm$ 5	9 $\pm$ 2
2-MeC <sub>28</sub>	13 $\pm$ 2	13 $\pm$ 3
(Z)-9-C <sub>29:1</sub>	tr	tr
C <sub>29</sub>	6 $\pm$ 3	6 $\pm$ 1
(Z,Z)-7,11-C <sub>31:2</sub>	5 $\pm$ 1	3 $\pm$ 1
(Z)-9-C <sub>31:1</sub>	9 $\pm$ 7	10 $\pm$ 2
C <sub>31</sub>	tr	tr
(Z,Z)-7,11-C <sub>33:2</sub>	1 $\pm$ 1	12 $\pm$ 3
Total hydrocarbon	730 $\pm$ 214	543 $\pm$ 156

<sup>a</sup> N = 5 groups of 10 flies each. (DB-5 column).<sup>b</sup> Trace is <1 ng/fly but detectable.

major fragmentation patterns indicated. A prominent molecular ion is found at  $m/z$  348 and diagnostic fragment ions at  $m/z$  152/153 and 166/167 for a double bond between carbons 5 and 6 and fragment ions at  $m/z$  250/251 and 264/265 for a double bond between carbons 9 and 10. Figure 2C is an EI-MS profile of what we have interpreted as (Z,Z)-7,11-C<sub>23:2</sub> obtained from *D. birchii*. The molecular ion at 320 is prominent, and a set of ions, equivalent to those in *D. serrata*, but shifted by 28 mass units is also prominent. We also found similar mass spectral evidence in *D. birchii* for trace amounts of (Z,Z)-9,13-C<sub>23:2</sub> and (Z,Z)-9,13-C<sub>29:2</sub> buried under the leading edge of the corresponding monoene peaks (Z)-11-C<sub>23:1</sub> and (Z)-9-C<sub>29:1</sub>, respectively.

TABLE 4. HYDROCARBON ABUNDANCE OF 5-DAY OLD *D. serrata*<sup>a</sup>

Hydrocarbon	Amount (ng/fly, mean $\pm$ SEM) <sup>a</sup>	
	Female	Male
(Z,Z)-5,9-C <sub>24:2</sub>	tr <sup>b</sup>	tr
(Z,Z)-5,9-C <sub>25:2</sub>	465 $\pm$ 85	379 $\pm$ 73
(Z)-9-C <sub>25:1</sub>	13 $\pm$ 3	12 $\pm$ 3
(Z)-7-; (Z)-5-C <sub>25:1</sub>	1 $\pm$ 1	14 $\pm$ 3
(Z,Z)-5,9-C <sub>26:2</sub>	tr	tr
(Z)-9-C <sub>26:1</sub>	tr	tr
(Z)-8-C <sub>26:1</sub>	tr	tr
(Z)-7-C <sub>26:1</sub>	tr	tr
2-MeC <sub>26</sub>	133 $\pm$ 13	101 $\pm$ 12
(Z,Z)-5,9-C <sub>27:2</sub>	tr	tr
(Z)-9-C <sub>27:1</sub>	9 $\pm$ 3	11 $\pm$ 2
(Z)-7-C <sub>27:1</sub>	tr	tr
(Z)-5-C <sub>27:1</sub>	tr	tr
(Z)-8-C <sub>28:1</sub>	tr	tr
(Z)-6-C <sub>28:1</sub>	tr	tr
2-MeC <sub>28</sub>	159 $\pm$ 22	109 $\pm$ 16
(Z,Z)-5,9-C <sub>29:2</sub>	tr	tr
(Z)-9-C <sub>29:1</sub>	tr	tr
(Z)-7-C <sub>29:1</sub>	tr	tr
2-MeC <sub>30</sub>	14 $\pm$ 2	7 $\pm$ 2
Total hydrocarbon	794 $\pm$ 129	633 $\pm$ 111

<sup>a</sup> N = 5 groups of 10 flies each.<sup>b</sup> Trace is <1 ng/fly but detectable.

At 5 days of age, there is hydrocarbon sexual dimorphism in the greater abundance of (Z)-11-, (Z)-9-, and (Z)-7-C<sub>23:1</sub>; (Z,Z)-7,11-C<sub>27:2</sub>; and (Z)-9-C<sub>27:1</sub> on *D. birchii* females (Table 3). *D. serrata* females had greater abundance of (Z,Z)-5,9-C<sub>25:2</sub> and the 2-methyl alkanes (2-MeC<sub>26</sub>, 2-MeC<sub>28</sub>, and 2-MeC<sub>30</sub>), whereas males had greater abundance of (Z)-7- and (Z)-5-C<sub>25:1</sub> (Table 4). A summary of shared hydrocarbon components between the two species and their relative abundances for 4-day-old virgin adults is given in Table 5.

**Female Choice for Male Cuticular Hydrocarbons.** Single classification multivariate analysis of variance (MANOVA) indicated that chosen and rejected males differed significantly across the nine hydrocarbons ( $F_{8,238} = 93327$ ,  $P < 0.001$ ). A canonical discriminant analysis was then used to determine a univariate description of cuticular hydrocarbons that best distinguished between chosen and rejected males. Since the canonical discriminant analysis was conducted on logcontrasts, to determine the contributions of individual hydrocarbons to the canonical variate that distinguished between chosen and rejected males, the

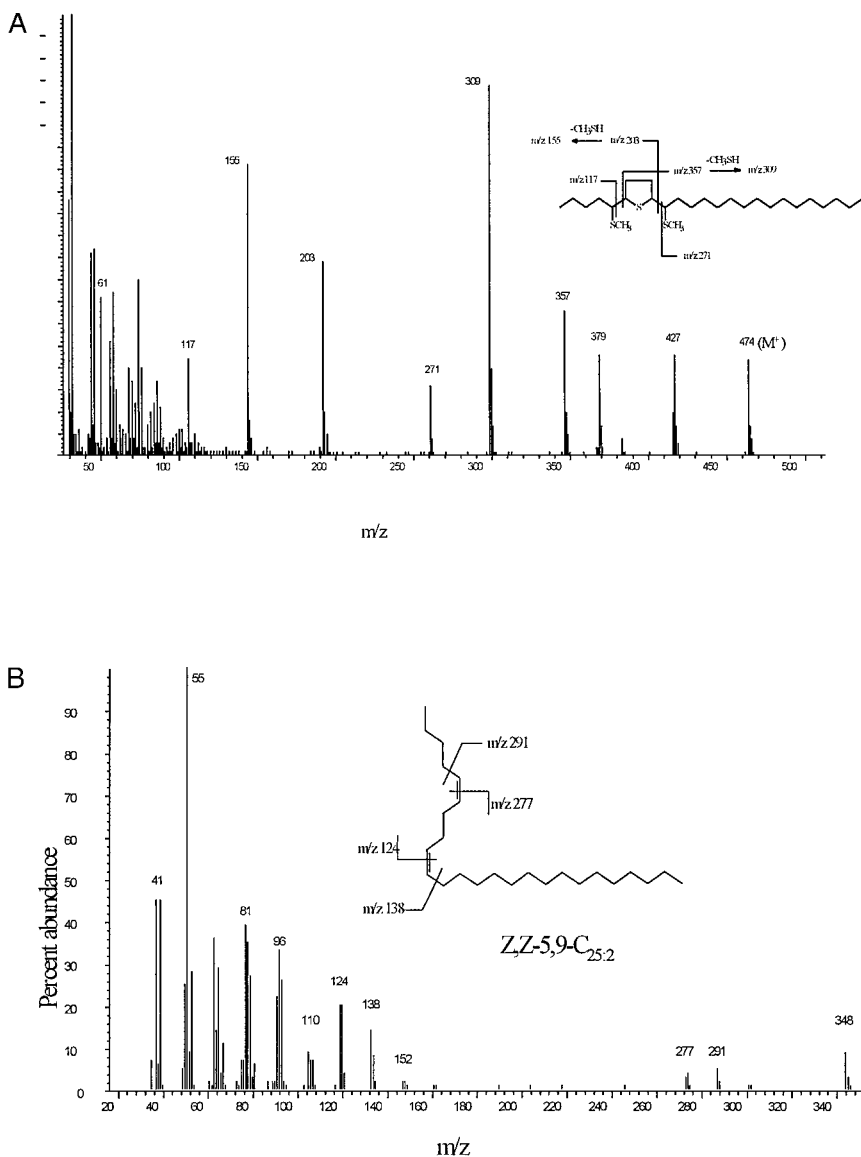


FIG. 2. (A) EI-MS of one of the heterocyclic reaction products resulting from the reaction of  $(Z,Z)$ -5,9- $C_{25:2}$  with dimethyldisulfide. (B) EI-MS of  $(Z,Z)$ -5,9- $C_{25:2}$  from *D. serrata*. (C) EI-MS of  $(Z,Z)$ -7,11- $C_{23:2}$  from *D. birchii*.

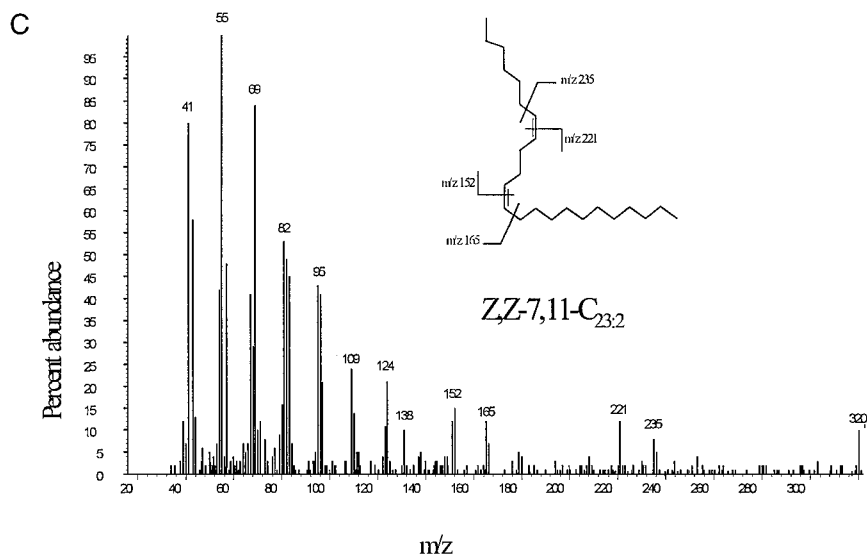


FIG. 2. CONTINUED.

canonical variate was correlated with the relative abundances of each of the nine hydrocarbons to generate a structure matrix (Table 6). Seven of the nine hydrocarbons displayed strong correlations between their relative abundance and the canonical variate. Of the nine hydrocarbons, the three 2-methylalkanes displayed elevated levels on chosen individuals, although 2-Me- $C_{26}$  was not significantly different between chosen and rejected males as indicated by the paired  $t$  test. In addition,

TABLE 5. SHARED HYDROCARBONS BETWEEN  
*Drosophila birchii* AND *D. serrata*

Hydrocarbon	Relative hydrocarbon abundance <sup>a</sup>	
	<i>D. birchii</i>	<i>D. serrata</i>
(Z,Z)-5,9- $C_{25:2}$	trace	AA
(Z)-9- $C_{25:1}$	AA	A
(Z)-7- $C_{25:1}$	A	trace
(Z)-5- $C_{25:1}$	A	trace
(Z)-9- $C_{27:1}$	A	A
(Z)-7- $C_{27:1}$	trace	trace
2-Me- $C_{28}$	trace	AA
(Z)-9- $C_{29:1}$	A	trace

<sup>a</sup> Trace is <2%; A is ca. 2–10%; AA is >10%.

TABLE 6. STRUCTURE MATRIX OF CANONICAL DISCRIMINANT ANALYSIS DISTINGUISHING BETWEEN CHOSEN AND REJECTED MALES

Hydrocarbon	Structure coefficient <sup>a</sup>	Paired <i>t</i> test
(Z,Z)-5,9-C <sub>24:2</sub>	-0.408***	<i>t</i> <sub>122</sub> = -2.584, <i>P</i> = 0.011
(Z,Z)-5,9-C <sub>25:2</sub>	-0.562***	<i>t</i> <sub>122</sub> = -3.591, <i>P</i> < 0.001
(Z)-9-C <sub>25:1</sub>	-0.363***	<i>t</i> <sub>122</sub> = -2.238, <i>P</i> = 0.027
(Z)-9-C <sub>26:1</sub>	-0.019	<i>t</i> <sub>122</sub> = -0.069, <i>P</i> = 0.945
2-Me-C <sub>26</sub>	0.289***	<i>t</i> <sub>122</sub> = 1.667, <i>P</i> = 0.098
(Z,Z)-5,9-C <sub>27:2</sub>	-0.128*	<i>t</i> <sub>122</sub> = -0.756, <i>P</i> = 0.451
2-Me-C <sub>28</sub>	0.519***	<i>t</i> <sub>122</sub> = 3.312, <i>P</i> = 0.001
(Z,Z)-5,9-C <sub>29:2</sub>	0.419***	<i>t</i> <sub>122</sub> = 2.315, <i>P</i> = 0.022
2-Me-C <sub>30</sub>	0.326***	<i>t</i> <sub>122</sub> = 1.999, <i>P</i> = 0.048

<sup>a</sup> \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001.

(Z,Z)-5,9-C<sub>29:2</sub> was elevated on chosen individuals and lower abundances of (Z,Z)-5,9-C<sub>24:2</sub>, (Z,Z)-5,9-C<sub>25:2</sub>, and (Z)-9-C<sub>25:1</sub> were found on chosen individuals (Table 6).

## DISCUSSION

*Cuticular Hydrocarbons of Drosophila.* The epicuticular hydrocarbons of the species-rich genus *Drosophila* differ qualitatively and quantitatively from the majority of insect species in having a relatively small number of hydrocarbon components (usually fewer than 30), whereas most insects examined have upwards of 100 or more (Howard, 1993). In addition, compared to other insects, a relatively small number of classes of hydrocarbons are associated with *Drosophila* spp. Unlike the majority of insects, *n*-alkanes are often found in only small amounts in any given species. 2-Methyl alkanes (and occasionally 3-methyl alkanes) are usually minor components (although the 2-methyl alkanes can be the most abundant class (Blomquist et al., 1985; Jallon and David, 1987), and only occasionally are internal methyl branched alkanes found in more than trace quantities. Instead, alkenes and alkadienes, and in rare instances alkatrienes are the dominant components of *Drosophila* hydrocarbon mixtures. Most of the unsaturated compounds have proven to be straight-chain compounds, with the exception of *D. mojavensis* where 2-methyl alkenes were found (Toolson et al., 1990). Qualitative and quantitative sexual dimorphism in hydrocarbon composition occurs for many but not all species (Bartelt et al., 1986; Jackson and Bartelt, 1986; Jallon and David, 1987; Toolson and Kuper-Simbron, 1989). Ontogenetic, temperature, and diet-related variation in *Drosophila* cuticular hydrocarbon profiles are also well known (Jackson et al., 1981; Jackson and Bartelt, 1986; Toolson et al., 1990; Markow and Toolson, 1990; Stennett and Etges, 1997).

Although numerous cuticular hydrocarbon studies have been conducted on various *Drosophila* sp. groups, many of them have been concerned primarily with major components that are thought to function as pheromonal signals [although undoubtedly, these hydrocarbons also serve an important role in desiccation resistance (Gibbs, 2002)]. The other hydrocarbon components are often either not described, or they are mentioned in only a cursory fashion with no supporting chemical or analytical data. This, of course, makes it difficult to make detailed comparisons among species and species groups. Despite this, the hydrocarbons of *D. birchii* and *D. serrata* can be partially contrasted with the various subgroups that have been previously examined. The most striking comparisons seem to be among the dienes. The *melanogaster* subgroup often has 5,9- and 7,11-dienes (Jallon and David, 1987), as does *D. birchii* and *D. serrata*. As young adults, *D. pseudoobscura* (*pseudoobscura* subgroup) possesses 5,9- and 7,11-dienes, but older adults possess 9,18-, 9,20-, and 11,20-dienes (Blomquist et al., 1985). *Drosophila mojavensis* and its sibling species *D. arizonae* and *D. navojoa*, which comprise the *mojavensis* subgroup, also have dienes with the double bonds nearly symmetrically placed at opposite ends of the chains (7,25-, 7,27-, 8,26-, and 8,28-), as well as possessing methyl alkenes (2-methyl with the double bond towards the other end of the chain) (Toolson et al., 1990; Etes and Jackson, 2001). In contrast, members of the *virilis* subgroup are characterized by 5,13-, 5,15-, and 7,15-dienes (Bartelt et al., 1986).

Despite being closely related, hydrocarbon compositions of *D. serrata* and *D. birchii* were strikingly dissimilar and may well be important components of the mechanisms providing species isolation between these two sympatric species. Of the 34 *D. birchii* compounds and 21 *D. serrata* compounds, only eight hydrocarbon structures were shared (see Table 5). Of those eight, only two, (Z)-9-C<sub>27:1</sub> and (Z)-7-C<sub>27:1</sub>, were similar in relative abundance. Low quantities of 2-methyl C<sub>28</sub> were also found in *D. birchii* males and females, but none of the other 2-methyl alkanes found in *D. serrata* males and females were found in *D. birchii*.

*Sexual Selection on Cuticular Hydrocarbons in D. serrata.* *D. serrata* females display strong directional choice for male cuticular hydrocarbons. At least four hydrocarbons are preferred in higher relative abundances, although we cannot exclude smaller potential effects from additional hydrocarbons. Of these four, 2-methyl C<sub>28</sub> is the most abundant and is also found in trace amounts on the cuticle of *D. birchii*. The other three contribute less than 5% each to the total cuticular hydrocarbon content on the *D. serrata* cuticle. Individual hydrocarbons that represent major proportions of total abundance are often the subject of study and have been associated with behavioral roles in other *Drosophila* species (Antony and Jallon, 1982; Jallon and David, 1987). *D. serrata* provides an example of how some minor components of the cuticular hydrocarbon profile of individuals (especially the 2-methyl alkanes) may have important roles in mate choice within species. It is not clear what sort of receptor mechanisms are involved or what sort of central nervous system processing would be required to make these distinctions,

but clearly the females are doing so. We note that recognizing the importance of these minor compounds using standard univariate statistical approaches may have been difficult and suggest that multivariate approaches to hydrocarbon statistical analysis should possibly be more widely used.

Cuticular hydrocarbons seem to be involved in recognition, in both species and mate choice within species in the *D. serrata* complex. Similar associations between levels of mate recognition have been observed in songs of some *Drosophila* (Ewing and Miyan, 1986), but not in head width in stalk-eyed *Drosophila* (Boake et al., 1997). However, it remains to be determined whether the same individual hydrocarbons are involved in both functions. If different hydrocarbons are involved that are genetically independent, the evolution of species recognition may be able to evolve independently from how individuals choose mates from among their own species. Alternatively, if the same hydrocarbons are involved in sexual selection within species and sexual isolation between species, then a direct role for sexual selection in the generation of reproductive isolation becomes more likely.

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